The Erythroblastic Island Niche: Modelling in Health, Stress and Disease.

Experimental Hematology

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--Manuscript Draft--

Manuscript Number:	20-179R1
Article Type:	Review Article
Keywords:	erythroblastic island; erythropoiesis; in vitro modelling
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Abstract:	Erythropoiesis is one of the most demanding processes in the body, with over two million red blood cells produced every second. Multiple hereditary and acquired red blood cell disorders arise from this complex system, with existing treatments effective in managing some of these conditions but few offering a long-term cure. Finding new treatments relies on the full understanding of the cellular and molecular interactions associated with the production and maturation of red blood cells, which takes place within the erythroblastic island niche. The elucidation of processes associated within the erythroblastic island niche in health and during stress erythropoiesis has relied on in vivo modelling in mice, with complexities dissected using simple in vitro systems. Recent progress using state of the art stem cell technology and gene editing has enabled a more detailed study of the human niche. Here, we review these different models and describe how they have been used to identify and characterise the cellular and molecular pathways associated with red blood cell production and maturation. We speculate that these systems could be applied to modelling red blood cell diseases and finding new druggable targets, which would prove especially useful for patients resistant to existing treatments. These models could also aid in research into the manufacturing of red blood cells in vitro to replace donor blood transfusions, which is the most common treatment of blood disorders.

Letter of Submission

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1st September 2020

Dear Connie,

Ms. No. 20-179

Thank you for your positive response to our Review manuscript on The Erythroblastic Island Niche: Modelling in Health, Stress and Disease.

We now submit a revised version of the manuscript where we have addressed all the comments of the reviewers and highlighted the key changes in red font. We include a detailed response to the reviewers' comments where we have outlined the key changes we have made and refer to the page and line numbers on the revised manuscript where appropriate. We have made minor alterations to the Figure and Table in response to the comments and now include a Graphical abstract. The reviewers' comments have been really helpful and have certainly served to improve the quality of our manuscript.

Please let me know if you require any further changes.

Yours sincerely

Lesley Forrester

Lesley Forrester
Professor of Stem Cell Differentiation

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Title: The Erythroblastic Island Niche: Modelling in Health, Stress and Disease.

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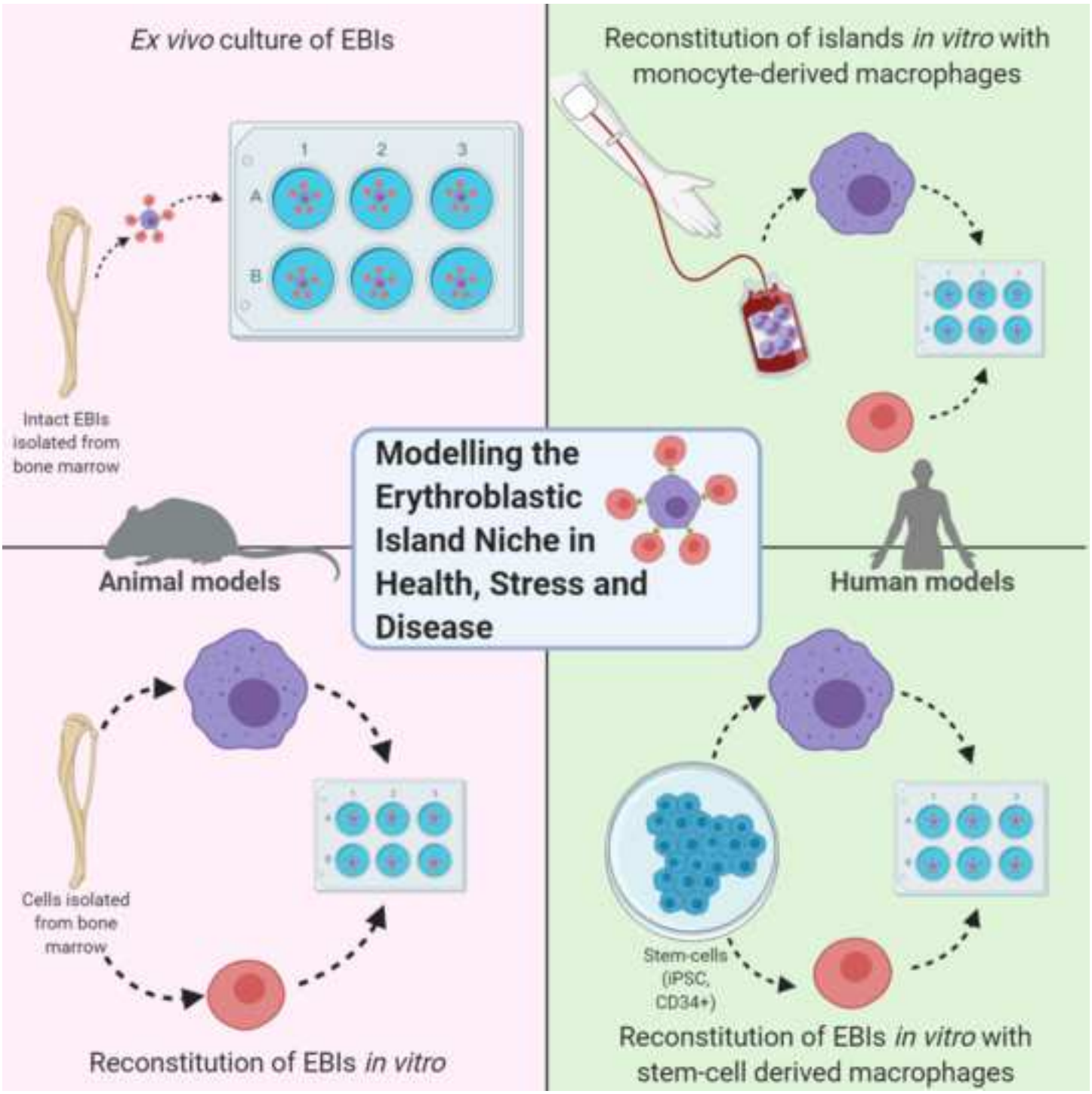
Word count: 4743

Graphical Abstract (for review)



Highlights

- Erythroid cells mature in association with macrophages within erythroblastic islands.
- Molecular interactions within the erythroblastic island niche are poorly understood.
- *In vivo* genetic models and *in vitro* co-culture systems have been used to gain insight.
- We reviews these different model systems and highlight the key findings to date.

The Erythroblastic Island Niche: Modelling in Health, Stress and Disease.

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Abstract

Erythropoiesis is one of the most demanding processes in the body, with over two million red blood cells produced every second. Multiple hereditary and acquired red blood cell disorders arise from this complex system, with existing treatments effective in managing some of these conditions but few offering a long-term cure. Finding new treatments relies on the full understanding of the cellular and molecular interactions associated with the production and maturation of red blood cells, which takes place within the erythroblastic island niche. The elucidation of processes associated within the erythroblastic island niche in health and during stress erythropoiesis has relied on *in vivo* modelling in mice, with complexities dissected using simple *in vitro* systems. Recent progress using state of the art stem cell technology and gene editing has enabled a more detailed study of the human niche. Here, we review these different models and describe how they have been used to identify and characterise the cellular and molecular pathways associated with red blood cell production and maturation. We speculate that these systems could be applied to modelling red blood cell diseases and finding new druggable targets, which would prove especially useful for patients resistant to existing treatments. These models could also aid in research into the manufacturing of red blood cells *in vitro* to replace donor blood transfusions, which is the most common treatment of blood disorders.

1. Introduction

The erythroblastic island, first visualised by Marcel Bessis in 1958, is the site of erythropoiesis in mammals (1). Erythroblastic islands are predominately situated in the bone marrow during steady-state erythropoiesis, but expand in the fetal liver and adult spleen during stress erythropoiesis when there is a rapid production of erythrocytes in response to inflammation and anemia (1-4). The island consists of a central macrophage (erythroblastic island [EBI] macrophage), surrounded by developing erythroblasts, which was hypothesised to supply ferritin to the developing erythroblasts for hemoglobin synthesis (5, 6). Additional functions of the central macrophage have since been elucidated, confirming its role as a supportive 'nurse' cell during erythropoiesis. EBI macrophages support erythroblast differentiation through cell-cell contact and secreted supportive factors, promoting the maturation and enucleation of erythroid cells and the phagocytosis of their expelled nuclei (7-10).

While there have been extensive studies into the hematopoietic stem cell (HSC) bone marrow niche microenvironment, the erythroblastic island niche (EBI niche) has been the focus of a relatively small number of research groups (11-13). In contrast to the vast array of cell types implicated in the HSC niche, the EBI niche appears to be relatively simple with no other cell type being implicated to work in concert with EBI macrophages (14). This offers the EBI as a unique and streamlined niche to study red blood cell (RBC) development in health and disease and the development of model systems is relatively straightforward.

Anemia represents the most prevalent RBC disorder and is crudely defined as a condition in patients who lack adequate numbers of healthy RBCs and diagnosed by a low blood hemoglobin (Hb) concentration (15). Anemia affects approximately 1.62 billion people worldwide, accounting for 8.8% of the total worldwide disease burden (16, 17). RBC disorders represent a broad spectrum of conditions, spanning inherited disorders such as thalassemia and sickle cell anemia, to acquired disorders such as polycythemia vera and paroxysmal nocturnal hemoglobinuria (PNH), with mild to fatal clinical outcomes (18-22).

Current treatments for RBC disorders include blood transfusions and iron chelation therapy to counteract iron accumulation in patients requiring repeated transfusion, or iron supplements for iron-deficiency anemia (23, 24). Several drugs that stimulate erythropoiesis have been approved, such as erythropoietin (EPO) that is used routinely to treat anemias associated with chronic kidney disease and cancer (25-27). Rarer RBC disorders, such as PNH, have fewer effective treatment options and these are often prohibitively expensive. Eculizumab, for example, is a monoclonal antibody against terminal complement protein C5 that is used to reduce hemolysis and stabilise hemoglobin levels. Not only is it widely reported as one of the world's most expensive drugs at a cost of around $400,000 per year of treatment, but only 20% of patients reach normal hemoglobin values and 40% remain anemic (28, 29). Some RBC disorders, including PNH and acquired aplastic anemia, can be effectively cured by bone marrow transplantation (30, 31) but this procedure is associated with significant morbidity and mortality rates due to the requirement for preconditioning which ablates the haematopoiesis of the host (31, 32). There is a clear clinical need for the development of new therapies to treat RBC disorders.

A full understanding of the cellular and molecular pathways involved in the EBI niche and how these contribute to stress erythropoiesis and disease would allow for the development of new therapies to treat RBC disorders. Early models of the EBI niche provided insights into its structure and organisation, later followed by more sophisticated genetic models that demonstrated the roles of individual genes in the niche in both animal models and humans. Here, we review the *in vivo* and *in*

vitro models that have been used to study the EBI niche. We discuss the advantages and disadvantages of each (Table 1) and summarise some of the key advances that have been made using these models in our understanding of steady-state, stress and disease erythropoiesis (Figure 1).

2. Erythropoiesis and the EBI

In the first stage of RBC production, HSCs sequentially differentiate to common myeloid progenitor, megakaryocyte-erythroid progenitor, burst-forming unit-erythroid (BFU-E), and colony-forming unit-erythroid (CFU-E) progenitor cells (33-36). In the second stage, CFU-E progenitor cells differentiate through the morphologically distinct nucleated precursors proerythroblast, basophilic erythroblast, polychromatic erythroblast and orthochromatic erythroblast (37). As the differentiating erythroid cells mature nuclear chromatin is condensed and cytoskeletal remodelling occurs in preparation for nuclear expulsion (38, 39).

The proerythroblast to orthochromatic stages of differentiation occur within the EBI. Macrophage-erythroblast adhesion molecules function to promote erythroblast proliferation and are highly expressed in proerythroblasts, with expression progressively lost by the orthochromatic erythroblast stage (40-42). The central macrophage secretes cytokines that promote the enucleation of erythroblasts, and provides iron for heme synthesis (6, 8, 43, 44). The composition of EBIs varies slightly across species, with rat EBIs containing consistently around 10 erythroblasts per island compared to much more variable human EBIs, where 5 to 30 erythroblasts can be found surrounding the central macrophage (45, 46).

At the final terminal differentiation stage, the nucleus is expelled from the orthochromatic erythroblast and phagocytosed by the EBI macrophage (47). The resulting reticulocyte expels any remaining organelles and enters circulation (38). Considerable membrane remodelling then takes place to generate fully mature, biconcave erythrocytes (48, 49).

3. Modelling the EBI Niche using Animal Models

3.1. Isolation of intact EBIs

Early investigations into the structure and organisation of EBIs involved careful isolation and study of rodent bone marrow using various types of microscopy (50). The first image of an EBI, obtained by Bessis in 1958, was achieved by examining bone marrow preparations using phase-contrast microscopy (1). Light and electron microscopy were later used to construct three-dimensional reconstructions of rat bone marrow, showing distinct in situ EBIs in which erythroblasts underwent maturation in close association with macrophages (2). After these initial morphological assessments of the central mononuclear cells present in EBIs as macrophages, F4/80 antibody staining confirmed that resident macrophages in mouse bone marrow formed the EBIs (51). Light and electron microscopy has also been used to demonstrate that EBIs are not spatially restricted within the bone marrow and their composition is altered depending on their location. For example, EBIs adjacent to sinusoids are enriched for orthochromatophilic erythroblasts, while nonadjacent EBIs are enriched in proerythroblasts, suggesting a mechanism in which islands migrate to sinusoids as erythroid differentiation progresses (52). Although the information gleaned from microscopy studies tends to present EBIs as static structures, the fact that they migrate indicates that they are actually quite dynamic and it is likely that the cell-cell interactions change over time. In tandem with microscopy studies, isolated EBIs were further characterised via antibody staining (53). EBIs were found to range from 5-100 cells, and the majority of isolated islands contained at least one F4/80^{+} central macrophage (53). Isolation of intact EBIs from different hematopoietic tissues and subsequent

analysis of known EBI macrophage cell surface markers revealed them to be a heterogeneous macrophage population (54-56). Mouse and rat EBIs heterogeneously express VCAM-1, F4/80 and CD169, while CD163 was only expressed by rat macrophages (54). The study of intact EBIs provided visual data for the composition of islands and is useful for characterising the component phenotypes of the EBI niche *in vivo* but it has limitations. It is a static system and cannot be used to dissect the molecular mechanisms associated with its function (Table 1).

3.2. *In vitro* reconstitution of EBIs

As well as *ex vivo* culture of EBIs harvested from rodents, the reconstitution of EBIs *in vitro* through the co-culture of erythroblasts and macrophages was first proposed in 1979, and is now a method widely employed (9, 42, 57-60). Through studies using reconstituted EBIs it was observed that macrophages support erythroid differentiation through their direct contact with erythroblasts via an EPO-independent mechanism, with cultured erythroblasts proliferating 3-fold more when in contact with macrophages (41, 42).

Studies employing reconstituted EBIs have been used to elucidate proteins involved in erythroblast and macrophage interactions. Erythroblast-macrophage attachments within the EBI niche are important in promoting erythropoiesis, and the adhesion proteins that facilitate these attachments are critical for island integrity. One of the first proteins identified to be involved in erythroblast-macrophage attachment was α4β1 integrin on erythroblasts that binds VCAM-1 on macrophages (55). Blocking monoclonal antibodies against α4β1 integrin and VCAM-1 significantly impaired erythroblast-macrophage attachment, demonstrating that this interaction is critical for island integrity (55). Reconstitution of EBIs *in vitro* has been a powerful model to observe the dynamic relationship between cell types, but the strategy is limited by culture conditions and does not allow for investigation of the possible interactions of other cell types in the bone marrow. Furthermore, it is unclear how this relates to the *in vivo* situation and how transferable it would be to the human EBI. (Table 1).

3.3. Macrophage depletion models

3.3.1. Macrophage depletion models in stress erythropoiesis

Macrophage depletion has been a particularly useful model to investigate the role of the macrophage compartment of the EBI niche *in vivo*. Macrophages can be depleted by administering clodronate-encapsulated liposomes which are phagocytosed and induce apoptosis (61-63) or using the CD169-DTR mouse strain which expresses the human diphtheria toxin receptor (DTR) under the control of the endogenous *Siglec-1* (CD169) promotor (64). Macrophage depletion is relatively easy to achieve, and can be applied to the different genetic models (Table 1).

Macrophage depletion models have been used to study the contribution of EBI macrophages to stress erythropoiesis in addition to the contribution of microenvironmental cells, for example splenic endothelial cells secrete SCF that mediates stress erythropoiesis in response to myeloablation, bleeding and pregnancy (65). Macrophage depletion has been shown to severely compromise stress erythropoiesis, impairing recovery from anemia, acute blood loss and myeloablation. In a study in which macrophages were depleted using clodronate there was a significant reduction in reticulocytes and erythroid precursors in both the bone marrow (BM) and spleen (62, 63). Depletion of $CD169^{+}$ macrophages using the CD169-DTR mouse model impaired recovery from hemolytic anemia and acute blood loss, and this was associated with a reduced number of EBIs and erythroblasts in bone marrow (66). In two models of acute RBC reduction, phenylhydrazine induced anemia and acute blood loss, a delay in hematocrit recovery was observed. There was also a delay in

the recovery of erythroblast numbers in myeloablation following BM transplant and following challenge with the myeloablative agent 5-fluorouracil (66).

One hallmark of stress erythropoiesis is the shift from erythropoiesis in the BM to extramedullary sites such as the liver and spleen, and the generation of stress erythroid progenitors (SEPs)(4, 67-69). During recovery from anemic stress, CCL2 production recruits monocytes to the spleen, where they associate with SEPs and differentiate into red pulp macrophages (RPM) creating new EBIs (70). In steady state conditions RPMs, a population of tissue resident macrophages, contribute to maintaining erythroid homeostasis by phagocytosing senescent erythrocytes, recycling iron and degrading heme (71). These RPMs have also been observed to form EBIs following transplantation and myeloablation (72). Splenic RPMs secrete BMP4 which is essential for the recovery of erythroid cells following a bone marrow transplant induced model of myeloablation (66). Spleens of $CD169^{+}$ macrophage-depleted mice, which had impaired erythroblast recovery, expressed significantly less BMP4 and reciprocal transplantation studies identified splenic RPM as the source of this BMP4 (66).

3.3.2. Macrophage depletion models in disease

Macrophage depletion models have been used to implicate the macrophage compartment of the EBI niche in various RBC diseases. In a murine model of polycythemia vera (PCV), a disease in which there is excessive production of erythroid cells as a result of a point mutation in JAK2 ($JAK2^{V617F}$), blood hematocrit was normalised following depletion of $CD169^{+}$ macrophages (66, 73, 74). Using the *Jak2*$^{V617F/+}$ murine model of PCV, researchers demonstrated that clodronate-mediated macrophage depletion also normalised RBC numbers (63, 75). JAK2 is a component of the EpoR signalling cascade, and the results of these macrophage depletion studies imply that the hyperactive EpoR signalling caused by the $JAK2^{V617F}$ mutant protein within the macrophage compartment contributes to the disease phenotype in this model of PCV (74). The fact that the number of macrophages increase in response to EPO administration in control mice confirms that EpoR signalling is indeed active in macrophages as well as erythroid cells within the EBI (76) and this is further supported by the expansion of both erythrocytes and macrophages in mouse models of erythrocytosis (76).

Macrophage depletion also improved the phenotype of a mouse model of β-thalassemia, with mice treated with clodronate exhibiting increased hemoglobin and RBC numbers (63, 77). Therefore, macrophages have been demonstrated to influence disease phenotypes by both increasing and decreasing RBC numbers.

While macrophage depletion has been an effective method to elucidate the role of EBI macrophages in stress and disease erythropoiesis, having been demonstrated to deplete $CD169^{+}$ $VCAM^{+}$ EBI macrophages, EBI macrophages are not exclusively depleted (Table 1). It is unclear what contribution, if any, the depletion of other macrophage subsets has on erythropoiesis. Further studies to isolate and deplete only EBI macrophages are needed to fully elucidate their contribution to disease.

3.4. Genetically modified mouse models

With the advent of gene editing technology, the role of specific genes in the EBI niche could be assessed *in vivo* using genetically modified mice (Table 1). Targeted deletion of genes hypothesised to have an important role in EBIs was used to strengthen observations made in previous *in vitro* studies. For example, antibody inhibition studies *in vitro* identified that ICAM-4, a member of the intercellular adhesion molecule family expressed in erythroid cells, binds to α4 β1integrin and αv integrins (78). The subsequent production of ICAM-4- null mice confirmed that its interaction with αv integrins on macrophages is involved in maintaining EBI integrity (58). Islands were harvested

intact from ICAM-4-null mice or reconstituted *in vitro* and a significant decrease in reconstituted islands was observed in isolates from ICAM-4 null compared to WT mice, supporting the functional role of αv integrin in EBI macrophages (58).

Targeted gene inactivation using a gene trapping approach confirmed the *in vivo* function of Emp (erythroblast-macrophage protein), which had been previously implicated as an important mediator of erythroblast-macrophage attachment *in vitro* (41, 79). Emp null embryos die shortly after birth and present with an increased number of nucleated, immature erythrocytes in their peripheral blood (80). The phenotype is especially stark in the fetal liver, where almost no EBIs were observed. Interestingly, control, wild type macrophages were still able to bind Emp-deficient erythroblasts but these erythroblasts failed to enucleate. Further insight into macrophage/erythroid interactions via Emp was gleaned from a macrophage-specific conditional gene deletion of *Emp (Maea)* that resulted in severely impaired EBI formation, whereas deletion in the erythroid lineage had no detrimental effect (81). Together, these studies indicate that Emp regulates the maintenance of macrophages and that Emp-mediated adhesion to erythroblasts must involve another currently unidentified receptor on erythroid cells (81).

Gene targeting studies in mice have also addressed the role of EBI macrophages in promoting enucleation via cell-cell attachments and in its function to phagocytose nuclei extruded from the erythroblasts. Nuclei are extruded as 'pyrenocytes', small, nucleated cells with a cytoplasmic ring (9). Pyrenocytes externalise phosphatidylserine providing a signal to macrophages for engulfment (47). The endonuclease DNaseII (Dnase2) in EBI macrophages destroys the nucleus expelled from erythroblasts as demonstrated in DNaseII-deficient mice, where undegraded DNA stimulates EBI macrophages to express IFN-β, therefore inhibiting erythropoiesis (82). It was particularly interesting to note that expression of *Dnase2a* in EBI macrophages is regulated by KLF1 (7, 83), a transcription factor that was first identified as a master regulator within the erythroid lineage, with an important role in regulating the later stages of RBC production including globin switching to the activation of erythroid-specific genes (84-87). Adding to its critical intrinsic role, an extrinsic role for KLF1 in the macrophage compartment of the erythroblastic island niche was first implicated using a KLF1-eGFP reporter mouse strain where GFP was detected in EBI macrophages and genes associated with island integrity such as VCAM1 expressed at a higher in KLF1-GFP^{+} macrophages (8, 88, 89).

Stress erythropoiesis has been shown to be particularly sensitive to modifications in the EBI niche, and mouse genetic models have also been used to assess the role of specific genes in that process (4). Steady state and stress erythropoiesis are thought to be regulated by different mechanisms with EBI macrophages being more strongly implicated under stress conditions. Growth-differentiation factor 15 (Gdf15), for example, is an essential regulator of stress, but not steady-state, erythropoiesis in both mice and humans (90, 91). *Gdf15*$^{-/-}$ mice exhibit a reduced expansion of the splenic stress erythroid niche from monocyte recruitment, resulting in an impaired proliferation of stress erythroid progenitors (SEPs)(91). It was demonstrated that Gdf15 signalling modulates Hif2α-dependent expression of BMP4 in macrophages through Vhl inhibition, which in turn regulates SEP proliferation (91).

Characterisation of the Epor-eGFP knockin reporter mouse demonstrated that the majority of EBI macrophages express the Epo receptor, implying that Epo can act on both macrophages and erythroid cells (56). It was recently shown that Epo/Stat signalling in splenic EBI macrophages represses the Wnt signalling that promotes SEP proliferation so the end result of macrophage Epo signalling is the differentiation of SEPs into functionally mature RBCs (92).

Stress erythropoiesis can also be induced during inflammation, where bone marrow hematopoiesis favours the production of innate immune effector cells at the expense of RBC production (93). Inflammation induced anemia is common in patients with chronic inflammation, and several pro-inflammatory cytokines, such as TNF-α and IFN-γ, have been shown to inhibit steady-state erythropoiesis (94-97). A recent study using a mouse model of sterile inflammation, demonstrated this inhibition is compensated by an increase in stress erythropoiesis. Signalling through Toll-like receptors (TLRs) stimulated the phagocytosis of erythrocytes by splenic macrophages, facilitating heme-dependent expression of the transcription factor SPI-C which in turn promoted the expression of both *Gdf15* and *Bmp4* that act to increase the proliferation of SEPs (98).

Could changes in the EBI niche contribute to RBC disorders as well as stress erythropoiesis, and if so, could modulation of the EBI niche be exploited to treat these RBC conditions? Experimental evidence in mouse models suggests that modulation of the EBI niche could be effective in treating RBC disorders. EPO, a hormone widely used to stimulate erythroblast production and maturation, is not always effective, with many anemic patients unresponsive to treatment (99, 100). Growth arrest-specific factor 6 (Gas6), a protein secreted by erythroblasts in response to EPO, enhances EPO signalling directly by activating the Akt survival pathway in erythroblasts, and indirectly by reducing the release of erythroid-inhibitory factors from macrophages in the EBI niche (101). In a *Gas6*$^{-/-}$ mouse model there is an increase in the expression of various cytokines known to inhibit erythropoiesis, for example IL-10, IL-13, and TNF-α (101). Thus, Gas6 could be a targeted as a potential therapeutic option that would act on both erythroblasts and EBI macrophages to promote erythropoiesis.

One major advantage of using genetic models is that, unlike *in vitro* models of the EBI niche, genetic models in mice has allowed for the dissection of the role of specific genes *in vivo*. However, they are much more expensive and complex than *in vitro* studies, and still may not accurately reflect the human system (Table 1).

4. Modelling the Human EBI Niche

The mouse models described have all contributed significantly to our understanding but how closely this resembles the human EBI niche is unclear. Work so far has focussed on the EBI macrophage, a key cell type in promoting erythropoiesis, but this has not allowed for the investigation into the involvement of other cell types (Table 1). Human EBIs can be isolated from bone specimens resected during surgery, for example ribs during thoracic surgery, or from sectioned bone marrow (45). Sourcing this tissue however is extremely difficult. Thus, the majority of studies into the human EBI niche have relied on *in vitro* modelling, mainly using cells sourced from peripheral blood. Recently, components of the EBI niche have been derived from either pluripotent stem cells or CD34^{+} hematopoietic progenitors and used to further characterise the human EBI niche (8).

4.1. Hematopoietic progenitor cell-derived macrophages

Modelling of the human EBI niche has been possible using CD34^{+}-hematopoietic stem and progenitor cells (HSPCs) that can differentiate into macrophages and erythroid cells. An Mpl-based Cell Growth Switch system was shown to drive macrophage-associated erythropoiesis and the macrophages produced in this system developed a phenotype similar to EBI macrophages; they expressed EMP, ICAM-4, CD163, DNASE2 and supported the maturation of erythroid cells to orthochromatic stage (102, 103).

The addition of the synthetic glucocorticoid, dexamethasone to differentiating CD34^{+} HPCs has been particularly useful in modelling human stress erythropoiesis *in vitro* (104). As erythroid

differentiation is inhibited by dexamethasone, the consequential expansion of pro-erythroblasts mimics the stressed situation. Interestingly the small population of macrophages that are generated under these conditions (3% of total culture) interact with the expanding erythroblasts to form EBIs. The presence of dexamethasone promoted the maturation of $CD169^+$ macrophages (104), the cell phenotype that is known to promote erythropoiesis under stress conditions in murine models (66). Taken together, evidence suggests that the EBI macrophages involved in stress erythropoiesis are likely to be distinct from those in the steady state.

4.2. Monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMCs) have been used to demonstrate that macrophages can function as a ferritin iron source for cultured human erythroblasts to synthesise hemoglobin, a long-suspected role of EBI macrophages (44). *In vitro* modelling of the human EBI niche has been consistent with findings obtained in the murine system demonstrating that contact with macrophages promotes erythroblast proliferation. Co-culture of $CD14^+$ PBMC-derived intermediate monocytes/macrophages with $CD34^+$ progenitors enhanced erythropoiesis by supporting progenitor cell survival (105) and the addition of glucocorticoids induced the differentiation of monocytes to macrophages with an EBI-macrophage phenotype (106).

Not only does this modelling rely on the availability of monocyte-derived macrophages, it is important to note that different effects have been reported on maturation and enucleation (63, 105). Furthermore, monocyte-derived macrophages may not accurately reflect the developmental ontogeny of EBI macrophages (107-110). To improve modelling of the human EBI *in vitro*, macrophages were derived from induced pluripotent stem cells (iPSCs) that can provide a limitless resource. iPSC-derived macrophages can be harvested repeatedly from cultures which greatly increases the yield of available macrophages compared to monocyte-derived (109, 111). Furthermore, iPSC-derived macrophages have been demonstrated to share ontogeny with *MYB*-independent tissue-resident macrophages, and while the exact developmental origins of EBI macrophages have not been determined, it is thought that they arise from yolk sac-derived EMPs, therefore sharing ontogeny with tissue-resident macrophages (108).

4.3. iPSC-derived macrophages

iPSC-derived macrophages were shown to promote the maturation and enucleation of RBCs differentiating from $CD34^+$ hematopoietic progenitor cells in an *in vitro* model of the human EBI niche (8). A tamoxifen-ER^{T2} expression system was used whereby the transcription factor KLF1 was expressed under the control of the constitutive CAG promoter and translocated to the nucleus upon addition of tamoxifen. Nuclear translocation and thus activation of KLF1 during the differentiation of iPSC-derived macrophages lead to the production of macrophages with an 'EBI macrophage' like phenotype. Macrophages expressing higher levels of KLF1 were better able to promote erythroblast maturation, resulting in an increase in enucleated RBCs in the culture (8). The use of this KLF1 expression system therefore supported previous findings of an extrinsic role for KLF1 in EBI macrophages (8, 89).

The use of this unique *in vitro* model of the human EBI enabled the identification of KLF1-regulated genes encoding secreted factor including IL-33, SERPINB2, ANGPLT7, that were shown to be important for promoting erythropoiesis (8). Addition of all three of these cytokines to differentiating $CD34^+$ haematopoietic stem and progenitor cell cultures significantly increased the absolute number of mature, enucleated cells, with removal of individual cytokines resulting in an overall reduction in the number of mature enucleated cells. Removal of IL-33 resulted in the most significant reduction

in mature cells, but IL-33 alone did not improve maturation implying that it acts in synergy with the other factors (8). This study demonstrated that *in vitro* modelling of the EBI niche using iPSC-derived macrophages provides a powerful tool to identify and characterise novel factors and the key signalling and regulatory pathways involved in erythropoiesis. It will be possible to use this *in vitro* model in future to assess the effects of these novel factors as therapeutic options for patients with RBC disorders. For example, cytokines such as IL-33 could be investigated for their ability to promote RBC proliferation and maturation in anemic patients who do not respond to EPO treatment and small molecules to activate or block key signalling pathways could be tested as potential therapies (99, 100).

The ability to genetically engineer iPSCs and thus differentiated macrophages presents a promising approach to dissect the role of individual genes within the human EBI niche (112, 113). The wide array of genetic tools also allows for controlled temporal activation and knockout of specific genetic pathways. The tamoxifen-ER^{T2} is particularly useful as it appears to recapitulate endogenous gene expression levels, avoiding very high, non-physiological expression levels often seen in standard transgenic or viral expression systems (8). The human iPSC-derived macrophage strategy also holds great potential in the context of RBC disorders, where pathogenic gene mutations can be introduced into EBI-like macrophages, to investigate whether genetic deficiencies in niche contribute to disease pathology.

In vitro modelling of the human EBI could also be used for patient-specific drug testing. This is especially useful for diseases in which animal models do not exactly recapitulate the disease. For example, the neonatal anemia, *nan* mouse carries a mutation (KLF1-E339D) homologous to the KLF1-E325K mutation that is observed in patients with type IV congenital dyserythropoietic anemia (CDA), but the phenotype is quite different (114, 115). Macrophages and RBCs differentiated from patient-derived iPSCs would allow for the *in vitro* modelling of EBIs in disease and could prove especially useful for dissecting the contributions of each cell type to disease pathology. Using an iPSC line derived from a CDA patient, it was shown that the KLF1-E325K mutation induces cell cycle arrest in differentiated erythroid cells (116). This was comparable to data derived from erythroid cells differentiated from patient-derived CD34+ progenitors where RNA-sequencing revealed the dysregulation of cell cycle genes (117). In future, the production of macrophages from this patient-derived iPSC line will elucidate whether the presence of the KLF1-E325K mutant protein in the EBI niche contributes to the pathology of the disease and will determine whether targeting the niche might be a therapeutic option.

5. Conclusion

The concept of the EBI has greatly progressed since it was first described by Bessis in 1958 with central macrophage functions, such as the role of cell-contact in promoting erythropoiesis, now well defined. *In vitro* models of the human and murine EBI, both intact and reconstituted, are relatively cheap and simple systems that have allowed for visual examination of the niche. Their simplicity however does not allow for dissection of the roles of individual genes, and in the human system sourcing primary tissue can be difficult (Table 1). While not possible in the human system, macrophage depletion and genetic models have been incredibly useful for understanding the role of individual genes within the mouse EBI niche. These genes are now beginning to be investigated in the human EBI niche, in which modelling using iPSC-derived macrophages now allows for the kinds of genetic manipulation employed to study the mouse system (Table 1).

The advances that have been made in modelling murine and human EBIs both *in vivo* and *in vitro* have great potential to be utilised to further examine the role of the EBI niche during stress

erythropoiesis and RBC disorders, especially by elucidating molecular mechanisms that could be targeted in their treatment. To recreate the EBI niche during stress erythropoiesis it will be interesting to assess whether iPSC-derived macrophages display the characteristics of splenic RPMs and if not, to devise culture conditions or genetic strategies to mimic their phenotype. The knowledge gained from the various models of the EBI niche could also aid in research to improve the production of functional RBCs from iPSC *in vitro* for clinical use. Blood transfusions, the most common treatment for RBC disorders, is reliant on donor supply and compatibility, and has side effects such as iron overload if patients require regular transfusions (118). Therefore, there is world-wide interest in the development of a donor-free supply of RBCs from a renewable source such as iPSCs. While significant progress has been made in this area, the strategy has not been successful in providing a stable source of enucleated RBCs that can be scaled up for therapeutic uses (119-123).

Acknowledgements.

Work in the author's laboratory is funded by the Medical Research Council (LF) and the Wellcome Trust (AM). We thank Niamh B McNamara for help with the graphics and Shyam Sushama and Antonella Fidanza for critical comments on the manuscript. Figure 1 was created with BioRender.com

Figure Legends

Figure 1. The role of EBI macrophages in erythropoiesis

In healthy steady-state erythropoiesis, erythroblastic island macrophages support and promote erythropoiesis via membrane protein interactions, the secretion of factors, and by providing iron for heme synthesis. During terminal differentiation, EBI macrophages phagocytose nuclei extruded from differentiating erythroid cells with DNAse11 associated with its degradation. In models of stress erythropoiesis, macrophage depletion led to the phagocytosis of mature RBCs by splenic red pulp macrophages, which increased expression of SPI-C, Gdf15 and Bmp4, resulting in an increase in the proliferation of stress erythroid progenitors. Macrophage depletion led to the normalisation of RBC numbers in disease models of polycythemia vera and β-thalassemia by decreasing and increasing red blood cells, respectively. Macrophages associated with the EBI in the steady state (purple) are likely to be different from those in stress and diseased conditions (blue).
(EBI**,** erythroblastic island; RBC, red blood cells; RPM, red pulp macrophages; SEPs, stress erythroid progenitors).

Table 1. Model systems used to study the erythroblastic island niche
Summary of the different mouse and human model systems used to study the erythroblastic island niche in erythropoiesis and their advantages and disadvantages. (EBI, erythroblastic island; HSPC, hematopoietic stem/progenitor cells; iPSC, induced pluripotent stem cells)

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May & Forrester (Ms. No. 20-179): Response to Reviewers

Reviewer #1:

1.Information by microscopy or even by FACS analyses of in vitro reconstituted islands are static and give the wrong impression that the island represents a stably associated structure. Macrophages, however, are very motile cells and in vitro time laps studies indicate that instead the island may be a dynamic structure possibly involving with time different cell populations. This dynamism may explain why some investigators are really skeptic that the island even exist.

We had included a reference that describes the migration of EIs (ref 52) but to emphasise this point we have now added a sentence to make it clear that the EBI is dynamic (Page 3, lines 38-41).

2. The review gives the impression that the same macrophages are involved in the formation of the erythroblast islands in steady state and in stress erythropoiesis. This may not be true. The macrophage that support CD34 survival derives from monocytes differentiated in culture. By contrast, the macrophages generated in cultures stimulated with dexamethasone that promote erythroblast proliferation likely derive from the stress erythroid progenitor cells which are generated in response to this hormone. These macrophages are likely to be stress specific because are not generated in cultures which do not contain dexamethasone (see Falchi et al, Haematologica. 2015 Feb;100(2):178-87. This paper inspired refs, 102). The fact that stress macrophages derive from the stress-specific erythroid progenitors explain their unique dependence on KLF1.

We have now added a separate section (Section 4.1) on CD34+ HPC-derived macrophages and the effect of glucocorticoids on their phenotype, citing the Falchi et al paper (ref 104) (Pages 7-8, lines 37-44 and 1-7). We have added a clear statement that EBI macrophages involved in stress erythropoiesis are likely to be distinct from those in the steady state. (Page 8, lines 6-7).

3. The Figure is good and clearly summarize the main concepts of the review. I suggest to modify the Figure to avoid the impression that the macrophage contained in the erythroblastic island under steady stare and stress/disease conditions is the same, which is probably not true. The niche involved in stress erythropoiesis is induced by glucocorticoids. In addition, I would spell out some of the factors that mediate the interaction between the macrophages and the erythroid cells in the various niches.

We have changed the colour of macrophages in Figure 1 to reflect the fact that macrophages in EI islands under steady state and stress/disease are likely to be different and modified the figure legend accordingly. We have added a new section on CD34+ HPC-derived macrophages where the addition of glucocorticoids mimicked stress conditions and make it clear that that EBI in steady state and stress conditions are different (Pages 7-8, lines 37-44 and 1-7). We have altered Figure 2 to include some of the specific membrane proteins that likely mediate the interaction between macrophages and erythroid cells under steady state conditions and we had added DNAseII within the macrophage which is involved in the degradation of the extruded nuclei (modified Figure 1).

Minor comments

We apologise for not including page and line numbers in the original manuscript. These are now included and we use these in our response for ease of referral.

- "completely ablates the hematopoietic system". Please change the sentence into "ablates the hematopoiesis of the host". After preconditioning, the resident macrophages in the bone marrow remain those of the host (Awaya et al Exp Hematol. 2002 Aug;30(8):937-42. doi: 10.1016/s0301-472x(02)00821-4).

We have altered the wording (Page 2, line 39)

- "such as the fetal liver and spleen". Since the authors talk about stress erythropoiesis in adult animals, they probably mean "such as the liver and spleen".

This mistake is corrected (Page 5, line 4).

- How an enhanced EPO receptor signaling via JAK2 may expand macrophages in polycythemia vera is not immediately clear. Do we know whether the resident macrophages in the bone marrow derive from the PV stem cell clone? This part needs to be clarified.

We have reworded this section to hopefully clarify the fact that hyperactivity of the EpoR signalling within the macrophage compartment is thought to contribute to the disease phenotype (Page 5, lines 20-26)

- The role of KLF1 in erythropoiesis should be better detailed/ made more precise. It is the last phase of terminal maturation (membrane-specific genes, nuclear condensation, etc) that is uniquely KLF1-dependent. The first part is mostly GATA1-dependent.

We have reworded this sentence to address the reviewer's point to "KLF1….with an important role in regulating the later stages of RBC production" (Page 6, line 24/25)

- "Prenocytes" should be pyrenocytes, In addition, this section requires some modification. Pyrenocytes are not naked nuclei extruded by the cell but real cells with a cytoplasmic rim generated by the last asymmetric division of terminal maturation. Since terminal maturation destroys the cellular cytoskeleton linking the nuclear and the plasma membrane and the mitosis motors, the two daughter cells must rely on physical contacts established by the pyrenocyte with the macrophage to provide the polarity force necessary to trigger their separation.

We apologise for the spelling error and have corrected this to pyrenocytes, we have added a description to clarify what they are and added a reference (9). (Page 6, lines18/19)

- Reference 4 (Paulson of 2011) should be replaced by the most recent review published by Paulson in Experimental Hematology in the last few months.

We thank the reviewer for alerting us to this reference and we have replaced ref 4 with this more recent review.

- Reference 18 is a review on PV by Tefferi of 2003. This reference should also be replaced by anyone of the more recent reviews on the subject.

We have replaced ref 18 with an updated 2019 review by the same author.

- Reference 112. Whether results published by Kohara with patient-derived iPCS are similar to those published by Varricchio et al using patient-specific erythroblasts differentiated in vitro should be discussed (Haematologica. 2019 Dec;104(12):2372-2380).

We have now included and cited the data described by Varricchio et al and note the similarities in the data generated from iPSC-derived erythroid cells and the patient CD34+ progenitor-derived cells described in that paper (Page 9, lines 27-29).

- For completeness, it is suggested to mention the contribution to the field of the Torok-Storb laboratory (Belay E et al PLoS One. 2017 Jan 30;12(1):e0171096, Pillai et al Exp Hematol. 2009 Dec;37(12):1387-92 and Belay et al Blood. 2015 Feb 5;125(6):1025-33.

We have now included and cited the work from this group (refs 102 and 103)(Page 7, lines 37-42). This is now included as part of a separate section on the generation of EBI macrophages from CD34+ hematopoietic progenitor cells and we have added this as a separate model in Table 1.

Reviewer #2:

1) The concept of EBI has greatly evolved since its inception several decades ago. Although the phagocytic activity of macrophages for processing the effete RBCs , or the pyrenocytes and supplying iron to the developing Erythroid cells were well accepted, other functions attributed to macrophages, i.e on the proliferation, on survival and terminal differentiation of erythroid cells were ill defined. In vitro experiments suggested contact dependent functions and these findings were supported by the results of antibody treatments. In vivo experiments addressed the dominant contribution of macrophages to stress responses, through Clodronate use(= a bomb with a lot of collateral damage),whereas the exact surface phenotype was constantly under revision. Along the way there were conflicting results, especially for some prior important players, i.e Spi-C ,or VCAM-1 or newer deletions of EMP in only erythroid cells or only in macrophages. Further, recently contact-independent functions of macrophages were emphasized through several secreted proteins. The current review cites all the authors prior conclusions, but needs a more critical evaluation of the findings.

We have now added text to the end of each section that includes a more critical evaluation of the models that are discussed (Page 4, lines 22-25; Page5, lines 33-36) and included a section at the start of our conclusions (Page 7, lines 22-25).

2) The contribution, especially to stress erythropoiesis, of microenvironmental (ME) cells other than macrophages, i.e endothelial cells, perivascular cells(secretion of SCF), dendritic cells, or the erythroid cells themselves, is insufficiently acknowledged. Upon high Epo stimulation erythroid cells release several molecules (Gas 6, VEGF, PIGF, GDF11) with paracrine effects on macrophages. Epo also elicits responses from macrophages, as indicated recently (the presence of Epo mRNA in macrophages was first reported in 1988!). So macrophages participate in a well coordinated response by many other cells types. Please add this.

We have added a sentence at the beginning of the macrophage depletion models in stress erythropoiesis section that addresses this point (Page 4 lines 34-37)

3) The review is subdivided into too many subchapters; i.e Clodronate use in normal in one and clodronate use in PV or Thal mice in another. Also on human EBIs, the monocyte-derived or iPSC-derived do not deserve a separate subchapter.
As our review is focussing on the different model systems that have been used to study the EBI, we believe it is important to define each model in a separately titled section.

Minor:
Ref #91 is incomplete and includes a wrong composition of authors.
Apologies, this reference is now complete

Table

Species	Model	Advantages	Disadvantages	References
Mouse	Imaging and isolation of intact EBIs	• Visual	• Static system	1, 2, 50
Mouse	Reconstitution of islands *in vitro*	• Allows the study of direct cell-cell interactions in a dynamic manner. • Cheap and simple	• not possible to investigate possible interactions with other cell types	42, 55, 57, 58, 59, 60
Mouse	Macrophage depletion *in vivo* (eg. clodronate)	• Easy to administer • Can be performed on different genetic models	• Broad macrophage depletion	63, 65
Mouse	Genetic models (eg lineage-specific knockout and depletion)	• Allows for dissection of the role of specific cell populations and genes *in vivo.*	• Costly • Complex	56, 58, 74, 77, 79, 80, 81, 83, 90, 100
Human	Imaging and isolation of intact EBIs	• Visual	• Static system • Difficult to obtain primary tissue	45
Human	Reconstitution of EBIs *in vitro* using HSPC-derived cells.	• Macrophage/erythroid interactions can be studied. • Relatively cheap and simple system • Macrophages and erythroid cells differentiate in concert	• Reliant on repeated donations • Does not investigate involvement of other cell types.	102,103,104
Human	Reconstitution of EBIs *in vitro* using monocyte-derived macrophages	• Macrophage/erythroid interactions can be studied. • Relatively cheap and simple system	• Not conducive to genetic manipulation • Reliant on repeated donations	44, 105, 106
Human	Reconstitution of EBIs *in vitro* using iPSCs-derived macrophages	• Macrophage/erythroid interactions can be studied • Relatively cheap and simple • Limitless resource • Genetically manipulatable • Disease-specific modelling possible	• May not completely recapitulate *in vivo* EBIs	8

Table 1. Model systems used to study the erythroblastic island niche
Summary of the different mouse and human model systems used to study the erythroblastic island niche in erythropoiesis and their advantages and disadvantages. (EBI, erythroblastic island; HSPC, hematopoietic stem/progenitor cells; iPSC, induced pluripotent stem cells)

Figure

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